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Effect of Two Bioformulation *Trichoderma harzianum* and *Pseudomonas fluorescens* with Manure in Controlling *Fusarium* wilt Disease in Pumpkin

Dhia S. Al – Waily **Sabreen M. Hassan**
Basrah University, Agriculture College, Plant Protection Dept.
Kararkarar603@yahoo.com

Abstract

This research aimed to isolate and identified the pathogen caused wilt in pumpkin plants and control by two bioformulation *Trichoderma harzianum* and *Pseudomonas fluorescens.*

The results revealed that the caused pathogen of pumpkin wilt plants was *Fusarium oxysporum* which diagnosis has been confirmed by using PCR Technique.

The results of pathogenesity revealed that 60.17% from Plants grown in contaminated soil infected with *F. oxysporum*. Also the results showed that use the *T. harzianum* that has high antagonism with pathogen, the degree of antagonism has reached 2 with *F. oxysporum* at Bell scale**.** *P. fluorescens* showed inhibition ratio against *F. oxysporum and T. harzianum.* reached 44 and 27% respectively. The results with use antibiosis for *T. harzianum* , *P. fluroescens* and manure revealed that the least severity was in the transactions MThPf (manure, fungi and Bacteria), MTh (manure and fungi), ThPf (fungi and bacteria), Th (*T. harzianum*), Pf (*P. fluorescens*), MPf (manure and bacteria), where ranged 0 - 5.56% , which did not differ significantly between them compared to M (manure) and control that reached 13.69% and 22.22% respectively**.** Weight in soil contaminated with pathogen in MThPf and ThPf that reached 376.83 and 379.22gr compard with control in the same soil when reached 66.41 gr .The results of the analysis with used GCMas revealed transactions in presence of Antibiotics and substances Phenol 2,5-bis (1,1-dimethyl), 4-Fluorobenzyl alcohol, Hexadecanoic acid (2) -methyl ester, 1-Pyrrolidine butanoic acid, gamma-Tocophenol and 9-Octanoic acid (2) -2-hydrox.

Key words: pumpkin, Cucurbita pepo, Fusarium wilt, Fusarium oxysporum, biological control, Trichoderma spp. , Pseudomonas fluorescens.

Introduction

Cucurbita pepo L . is one of the most important summer vegetable crops desired in Iraq and is cultivated in all areas of Iraq in the open fields. Planted by two seasons , the first one is spring season begins in March to give production in late April and the second autumn season during August to give production in October (1).

Each 100g of pumpkin fruits gave a low proportion of fat and carbohydrates reached 66% , protein 3% ,High carotidene content 15%. , and fiber 11.46% , ash 16% and 80 calories(2 and 3).

The symptoms of infection by *Fusarium oxysporum* in plants, the plant is wilted gradually with the yellowing of the lower leaves, symptoms of wilt may suddenly appear on the plants in the warm atmosphere or the sunny notes also notice a clear change in color of stem or in the holding of the leaves and when working a longitudinal section in the stems showed the vessels are colored brown (4).

(5) proved that *P. fluorescens* bacteria are capable of inhibition of the growth of the *F. oxysporum* in media and its ability to stimulate systemic resistance and reduced disease severity in pathogenicity. As a result of the lack of studies on the plants of pumpkin Therefore, the study aimed at isolating and diagnosing the caused pathogen

agent of pumpkin plants by PCR technique and then controlled by two bioformulations *Trichoderma harzianum* and *Pseudomonas fluorescens.*

Material and Methods

Isolation and diagnosis of caused pathogen

Random samples of the waxy plant pumpkin were brought to the lab from the Safwan and Zubair regions in the province of Basra, the infected plant parts (leaves) were washed with running water , cut by sterile scalpel (1 cm) and then sterilized with sodium hypochlorite solution (NaOCl) at 1% concentration for one minute , It was then washed with distilled water to remove the traces of sodium hypochlorite solution and dried with filter paper type whatman1, and then planted in the petri dishes which had 9 cm diameter contain solid PDA media which added antibiotic Chloramphenicol (250mg.l^{-1}) replicated with three dishes which had five pieces per dish, then incubate at 25 ± 2 ° C for 7days (6).

The diagnosis of pathogen by PCR Technique

The used of PCR procedure in Sciences faculty for girls University.of Babylon by Prof.Dr.Ziedan AL-Maamory .

The pathogenicity of *F. oxysporum* test:

F. oxysporum **w**as grow on millet grains and incubated at 25 ± 2 ° C for 14 days , which was added to(10gr/pot) the plastic pots (6 kg)which contained steriled soil and manure (1: 3), which replicated with three pots in addition to the control treatment, The four-week-old pumpkin seedlings were picked up in corks.

The severity of infection was calculated after four weeks according to the following pathological severity scale:-

Degree	Infection
1	Light yellowing and wetness of the first two leaves
2	Yellowing and wetting a number of lower leaves
3	Extreme wilt and plant death

An equation of Mickenny has been applied (6).

The fungi were then re-isolated from the plants that showed the infection.

The inhabition of *F. oxysporum* and *T. harzianum* with *P.fluorscens* .

The sterile medium KBA was infused in sterile plastic Petri dishes diameter of 9 cm and then the dishes were vaccinated with a bacterial KBA at 48 hours with four drops / dish of 0.1 ml / drop , the sides of the diagonals are perpendicular and 1 cm from the edge of the dish and then incubated the dishes at 25 ^{o}C for a period 48 hours , Infected the center of each dish 0.5 cm diameter from the *T. harzianum* which growth in PDA medium by four days' by three replicates with control treatment and then incubated at 25 ± 2° C. The percentage of inhibition after growth in the control treatment was calculated to the edge of the dish according to the equation Aboutt 1935(6) .

The effect of manure and bioformulation of *T. harzianum* and *P. fluorescens* in growth indicators.

After four-week the pumpkin plants were transferred to soil with organic fertilizer, biopharmaceuticals and pathogenic fungus in a field of 16 m long and 4 m wide in soil wich covered with plastic and added to the soil and installed hoses Doping was carried out and the treatments such as with biochemistry + bacteria + compost

(MThPf) and biochemistry + manure (MTh), Bacterium + manure(MPf), manure (M), Biochemistry + Bacteria ThPf, Th, Pf, and Control. (1) were carried out . The same treatments were performed without the use of the pathogen, and the research soil was divided into two parts: the first was added to the fungus *F. oxysporum* by 10 g / m long and the other part was left without the addition of pathogenic fungus Bioreactors were added 10 g / m in length (6).

Determination of some compounds in some treatments using GC-mass technique.

In the flowering stage, a group of leaves was randomly taken for each treatment well washed with water to remove the dust and then distilled water and dried the leaves at 70 ° C. for three days , crushed to fine powder using ceramic mortar and stored at 40 ° C ,It took 20 g of powdered hazelnut leaves for each treatment and placed in a kirin electric mixer and added 200 ml ethyl alcohol at a concentration of 99. 99% for 10 minutes , then put in mixture in a hotplate magnetic Stirrer for 48 hours at 50 ° C, And then placed in the centrifuge at 3000 cycles / minute for 30 minutes, and neglected the deposit and take leachate and repeated and nominated using the paper filter No. whatman1, which was placed in the oven at 40 º C (7) , and then placed in sterile bottles, and samples were analyzed at the environmental research center in Baghdad department of environmental research and water affiliated to the ministry of science and technology.

Result and Discussion

Isolation and diagnosis of *F. oxysporum* and pathogenicity:

Isolation of *F. oxysporum* from the pumpkin plants which infected with *Fusarium* wilt disease. The morphological characteristics, spores form, pregnant carrier and non-natural structures by Assist. Prof. Dr. Dhia Salem Al - Waily according to the taxonomic keys contained in (8) and (9), It has been observed that colonies of fungi characterized by lumbar growth or sparse or abundant fungal yarn with the emergence of rings Surrounding the mushroom center, colonies of this mushroom appear pale orange and sometimes appear in violet or the pale purple from the back of the dish, In addition to the fungus, there are three types of spores, which are large conidic spores that are short to medium in length straight to the sickle shape and small conidial spores in large numbers It is characterized by its semi-oval shape, which originates on the phyllides and often has a basal foot cell, the spines are spherical and sometimes curved. These spores consist of a group of short-term assemblies and chlamydia spores that appear either in single cells or in short-term or interstitial chains ,That's agree with (8) and (9) .

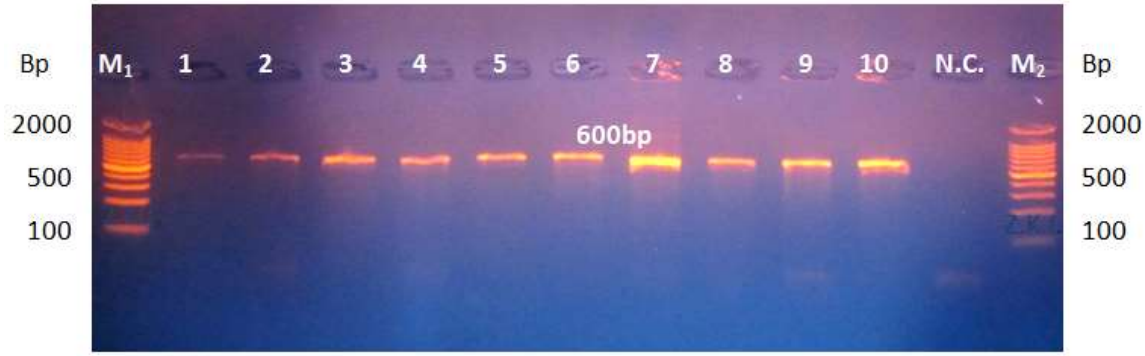

Fig.(1) Alkarose gel for polystyrene product for the ITS1-5.8S-ITS2 region using the PI5SIS / ITS4 primer pair of *F.oxysporum* isolates which isolated from the plant (10-1) which showed a hereditary pattern (BP 600) approximately N.C. Negative control. M1, M2 = Molecular parameter (2000bp) Each step = 100bp

The results(Fig.2) showed that the severity of *F. oxysporum* was 60.71% Compared to the comparison in which no injury occurred.

The symptoms of the infection appeared on the plant in a slight yellowing and the first two leaves wilt and then yellowing and the decline of a number of lower leaves and the progress of the fungus led to a severe wilt of the plant and the death of the entire plant .This may be due to the introduction of fungus into the plant tissue by penetrating the cellular walls due to susceptibility some isolates on the production of cellulose and decomposed enzymes for cell walls (10).

The pathogen fungi attack the root of the plant and grow in the interstitial spaces and eventually extend the innate spinning of the wooden veneer vessels and settle in. The vessel can be filled with fungal spores, small conidic bacteria or polysaccharides produced by fungi. The receptor of the vessel is increased from the products of the decomposition of plant cells by pathogenic fungus enzymes (11).

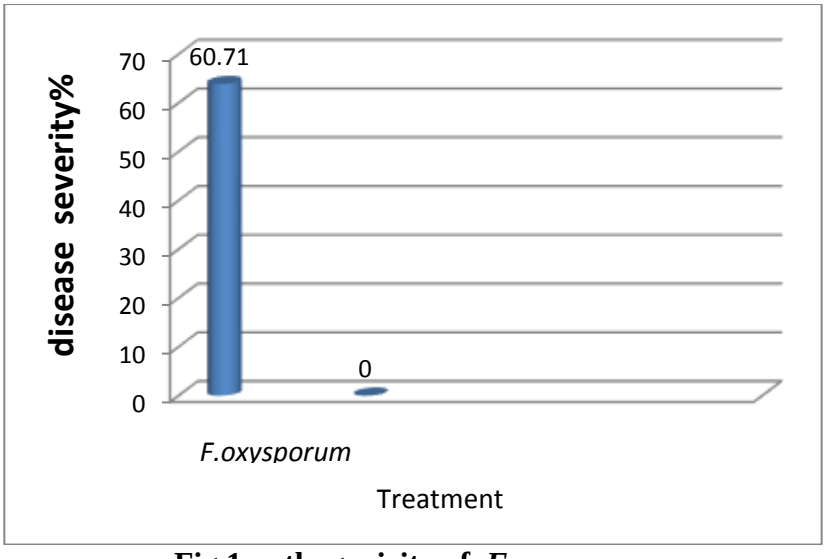

Fig.1.pathogenicity of *F.oxysporm.*

Antibiosis of *T. harzianum*. against *F. oxysporum.*

Results (Table 1)suggest that *T.harzianum* has high antibacterial ability with *F. oxysporum* where it worked to discourage it on the media , where the average growth rate of the colony 2.04 cm compared to the growth of fungus on the media alone, which reached 8.5 cm, as shown by the same table, the degree of antagonism was class 2 to inhibit pathogenic fungus, explaining the high efficiency of *T.harzianum* .

The path of the fungus on the basis of direct parasitism By spinning the yarn around the fungal threads of the nurse and grow on them , These agree with (12) he has been found that the mechanism used by the fungus resistance through microscopic observations in the dual farms(Double Culture) It includes the vital resistance fungus spinning snail (Coiling)around the pathogen and then the secretion of antibiotics and some enzymes to analyze the walls of fungus cells, such as Protease و β-1-3-glucanase وChitinase , This result confirms the findings of (13).they found the antibiotics and enzymes are found to assist in the analysis, feeding and feeding of host cells (Necrotrophic).This agree with (14) and (15), whom found that the fungus of biological resistance is used in several mechanisms, such as brooding on fungus, the secretion of antibiotics or competition for food and place.

Table1 : Antagonism between *T. harzianum* and fungi disease *F. oxysporum.*

Treatment	Colony growth rate (cm)	Degree of antagonism
T.harzianum+F. oxysporum	*2.04	2
F.oxysporum	8.5	

mean three replicates*

Inhibition of *F.oxysporum* and *T. harzianum* by *P. fluroscens.*

The results as showed in Table (2) indicate that *P. fluorescens* was inhibit *T. harzianum* by reached at 27% in PDA mediuum , *P. fluorescens* reduced the growth of *T.harzianum*. *P. fluorescens* showed high inhibition efficiency of *F.oxysporum* by reached at 44% which caused inhibit of fungi growth on medium , and may be due to the inhibition of *P. fluorescens* for fungus to produces some antibiotics such as lipopeptid , amphisin and their production of some enzymes surrounding the fungal cell walls the enzyme such as endochitinase (6 and *16*), this result agree with (17), he explained that *P. fluorescens* in the inhibition of the growth of *F.oxysporum* reached 100% .(5) explanted the ability of *P. fluorescens* to inhibit the growth of *F.oxysporum* where the highest percentage of inhibition compared to other tested bacteria rached 52% , (18) also demonstrated that the thermodynamic *Pseudomonas glumia* bacteria showed effective resistance against *P. solacearum* in the center of the plant,may be the high resistance of *P. fluorescens* to the production of different types of antibiotics such as phenzain -1-caroxylate (19) or to the production of enzymes for fungal cell walls such as Chitinolytic enzyme and Catalase and the production of HCN (20).and enzyme chitinaseylytic, Protease and b-1.3-glucanase (21).

Table 2: Effect of *P. fluorescens* in *F.oxysporum* and *T.harzianum*

Treatment	Inhibition %
F.oxysporum	44
T.harzianum	27

Effect of different treatments in the disease severity of the *F.oxysporum.*

The results showed (Table 3) that no difference between MThPf ,MTh ,MPf ,ThPf ,Th and Pf which recorded between 0- 5.56 %, when compared with treatments M (Manure) and control which recorded at 13.89 and 22.22 % respectivily. This result agree with (17) The ability of *P.fluorscens* to protect the plants from the infection with *F.oxysporum* and reduce the percentage and severity of injury due to the mechanisms used by bacteria inhibition, these mechanisms include the production of antibiotics such as phenazine-1-carboxylicacid, the production of pyoluteorin, HCN, growth-regulating hormones, chitinolytic enzymes, and catalase and producing Siderophores compounds (22) .Bacteria also produce different types of antibiotics such as Pyrrolnitin, 4Diacetylphloroglucinol and Siderophores (23) , or through the strong competition for food between the pathogen and the biological resistance factor, which leads to the removal of the pathogen from the root surfaces, which represent the appropriate environment or the effect may be caused by the effectiveness of bacteria in the induction of systemic resistance and stimulate the growth of plants as the bacteria slow the spread of the vaccine Fungal around the roots (24) showed that if time is needed to show the induced resistance in the development of the disease is its effect before the infection.

Table (3) Effect of different treatments on the severity (%) of infection in the pumpkin plants.

Treatments	%Infection
MThPf	0
MTh	0
MPf	56.5
Manure	89.13
ThPf	0
Th	78.2
Pf	78.2
Control	22.22
LSD 0.05	81.5

Effect of different treatments in plant lengths

The results showed in Table (4) that the mean treatment MThPf increase the length of the plants when recorded 286.49 cm compared with the control which reached at 70.00 cm. The soil Contaminated with *F.oxysporum* shows that have an increase in

plant height rates when reached 200.18 cm compard with the mean length of plants planted in contaminated soil, which amounted to 162.34 cm. The results of interaction in contaminated soil showed that the treatment MThPf increase the length of the plants, where the height was 253.05 cm compared to the control in the same soil when reached at 31.72cm. The reason for increased plant growth due to resistance treatment bacterial biology can be stimulated by plant growth due to its role in increasing nutrient elements (23) or stimulate the growth of the plant and thus strengthen its resistance to pathogens by the production of acid Salicylic acid, which stimulates systemic resistance in the plant as well as the newly recognized ability to stimulate the production of growth hormones Kalawksin and Jabrlin and increase Chlorophyll in leaves (24 ;.,25) or because of their role in stimulating systemic resistance and resistance to pathogens or within these combined mechanisms (26)This agree with (27). The positive effect of *T. rhizianum* which increased the dry weight of the root and vegetative mass and the plant-length rate to the effect of the bioconcentration factor in inhibiting the activity and inhibition of the pathogen on the one hand, as well as its role in the production of catalysts or regulators for plant growth or by increasing the nutrient elements such as phosphorus, iron and zinc (28).

Table 4 : Effect of different treatments on lengths of pumpkin plants in the soil contaminated or non - contaminated with *F.oxysporum* in the field .

Treatments	Plant length (cm)		Mean
	Soil contaminated with *F.oxysporum*	Soil with out *F.oxysporum*	
MThPf	253.65	283.93	268.49
MTh	218.72	253.14	235.93
MPf	189.63	216.64	203.14
Manure	58.89	129.34	94.12
ThPf	201.73	235.69	218.71
Th	201.97	210.39	206.18
Pf	142.72	164.04	153.52
Control	31.72	108.28	70.00
Mean	162.34	200.18	

$LSD_{0.05}$ of treatment = 36.15, for soil with or without *F.o* = 38.34, for interaction = 23.48

Effect of different treatments on the soft weight of the vegetative

The result (Table 5) shows that the mean treatment MThPf increased the weight of the plants when recorded 483.48 gr compared with control which reached at 104.59 gr Soils with out *F.oxysporuum* showed an increase in the plant weight 304.99 gr compared with average weight of plants cultivated in contaminated soil when reached at 232.04gr. The effect of the interaction between the treatments and their addition to the contaminated soil and uncontaminated showed the treatment of MThPf get an increase in the weight of the soft vegetative plants when recorded 590.12 gr compared with the control that reached at 66.41 gr.

This variation in effect may be due to the fact that sterile animal manure is an appropriate environment for fungus growth *T.harzianum* It is rapidly and densely settled as a result of its containment of nutrient materials and lack of competition for

other microorganism (29 and 30). As a result of the growth and proliferation of *T.harzianum* profusely in that environment his was clearly reflected in the indicators of growth and productivity of tomato through its secretion of certain substances such as Pyrones (31) or enzymes (32) or secretion of growth regulators (33) , this can be explained by the fact that sterile organic substances decompose and release large amounts of dioxide Carbon is thus the formation of a carbonic acid that increases the efficiency of photosynthesis and thus increases total vegetative. *T.harzianum* has a significant role in nitrogen, phosphorus and sulfur, which has the potential to increase the processing and absorption of nitrogen by the plant and plays an important role in the melting of trace elements Zn, Mn, Cu and Fe in soil conditions Basal and plant needs in growth and root formation (28) .

The researchers confirmed that the ability of bacteria to control the pathogen was not limited to control, but it is believed to have the ability to stimulate the enzymes responsible for the systemic resistance in the plant (34).

Table 5: Effect of different treatments in plant soft vegetative weights (gr) in soil contaminated or non - contaminated with *F.oxysporum* .

Treatments	Plant soft vegetative wieght (gr)		Mean
	Soil contaminated with *F.oxysporum*	Soil with out *F.oxysporum*	
MThPf	376.83	590.12	483.48
MTh	315.86	359.09	337.48
MPf	217.63	311.87	291.75
Manure	136.76	175.98	156.37
ThPf	379.22	403.36	.391.29
Th	146.56	190.16	168.36
Pf	163.05	266.62	214.84
Control	66.41	142.77	59.104
Mean	232.04	304.99	

LSD0.05 of treatment = 76.23 , for soil with or without *F.o* = 49.51,
For interaction = 120.45

Determination of some compounds in some treatments using GC-mass technique.

The results appeared as explaned in table (6) that the treatments MThPf , MTh ,MPf ,ThPf ,Th and Pf when analysis with used GCMas revealed transactions in presence of antibiotics and substances Phenol 2,5-bis (1,1-dimethyl), 4-Fluorobenzyl alcohol, Hexadecanoic acid (2) -methyl ester, 1-Pyrrolidine butanoic acid, gamma-Tocophenol and 9-Octanoic acid (2) -2-hydrox. Some phenolic compounds seemed by Al-daili(2017) in pumpkin when used *T.viride* as Ascorbic acid 2,6-dihexadecanoate-1-(+ 1,3-Propanediol,2-ethyl-2-(hydroxymethyl) و D:B-Friedo-B:A-neogammacer-5-en-3-l,(3:beta).

Table (6)Compounds in plants treated by some factors in contaminated soil.

Treatments	Compounds	Molecular weight
MThPf	Elaidic acid , Phenol,2,5-bis(1,1-dimethyl ethyl) isopropleste Silane diethylpentadecyloxy-13-phenol	55 84 33
MTh	4-Fluorobenzyl alcohol Methyl Methyl Tetradecanote 2-pentadecaneno-6-10-14-trimethyl Hexadecanoic acid – methyl ester	46 97 49 98
MPf	Cyclohexadecane,1,2-diethyl 9-Octdecenoic acid (2)-hydrox Gamma-Tocopherol	95 99 94
ThPf	Natolensine-3,5-dintrobenzoate Phenol-2,5-bis(1,1-dimethyl ethyl)	92 70
Th	Trichloromethan 9-Octadecanonic acid (2)-methyl ester Hexadecanoic acid – methyl ester	97 96 99
Pf	Cyclohexadecane ,1,2-diethyl 1-pyrrolidne butanoic acid 2-H-1-Benzopyran-6-1-3-4-dihydroxide	95 37 62

CONFLICT OF INTERESTS.

There are non-conflicts of interest.

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